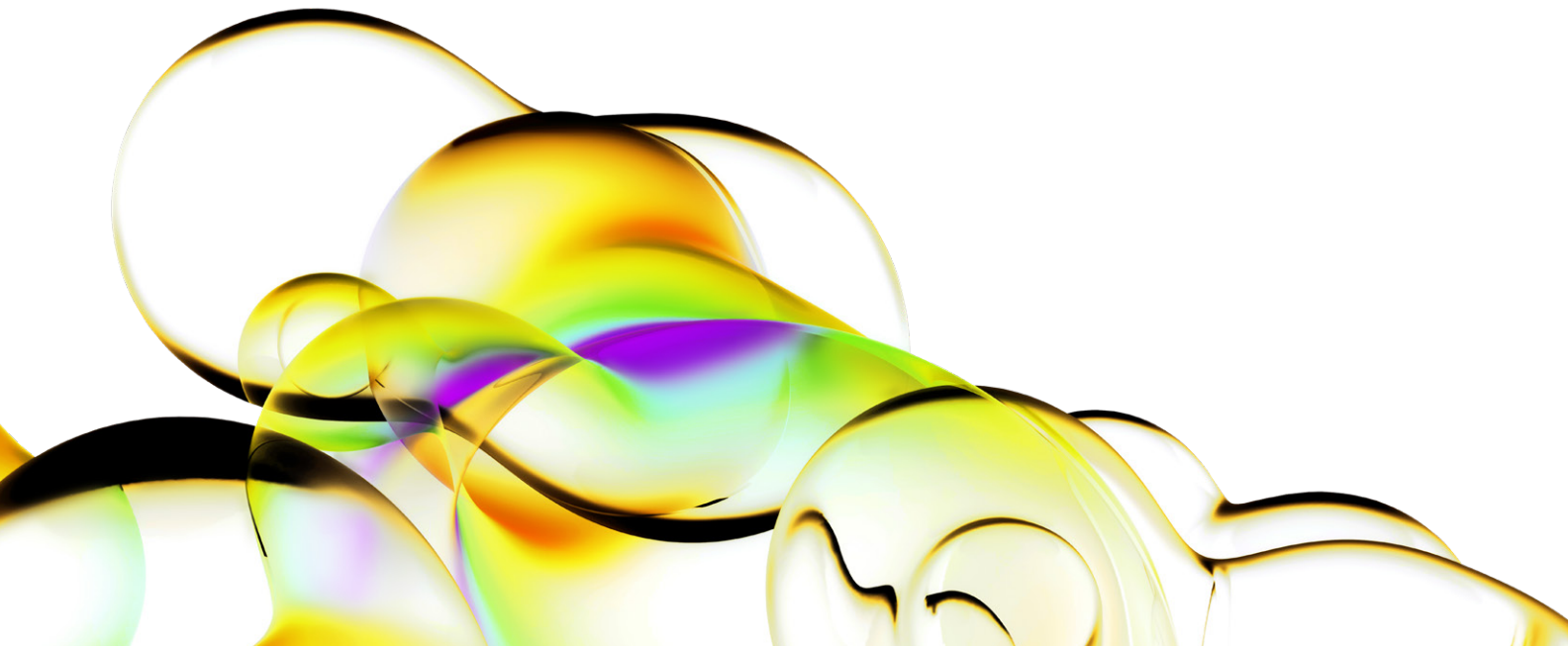
Time-course monitoring of primary murine B1 and B2 Cell proliferation using Cellometer image cytometer.

Introduction

Cell proliferation is an important assay for pharmaceutical and biomedical research to test the effects of a variety of treatments on cultured primary cells or cell lines [1, 2]. Previously, we have reported a rapid and accurate fluorescence-based cell population analysis method using a novel image-based cytometry system. The method is highly comparable to traditional flow cytometry using fewer cells [3-6]. Here we report the development of a novel method for the kinetic measurement of cell proliferation using the Cellometer® image cytometer.

Kinetic analysis was performed via fluorescent detection of carboxyfluorescein succinimidyl ester (CFSE)-labeled primary immune cells. To test if the Cellometer system can accurately detect proliferation of rare cell types, we selected B1 B cells, which are fewer in number than conventional B2 B cells. To validate the analysis method, proliferation results were compared to end-point analysis from flow cytometry. In order to demonstrate the capability of the detection method, a panel of Toll-like-receptor (TLR) agonists (Pam3cys, LPS, PolyIC, CLO97, and CpG), were used to study the proliferation effect on B1 B cells, where propidium iodide (PI) was used to determine cell viability at each time point [7-11]. The results discussed here demonstrate that the method has the potential to revolutionize kinetic analysis of rare cell samples like B1 B cells.



Materials and methods

Cellometer vision and disposable counting chamber

Cellometer instrumentation has been described previously [3]. The system utilizes one brightfield and two fluorescent optics modules, VB-535-402 (EX- 475 nm/EM-535 nm) and VB-660-502 (EX-540 nm/EM- 660 nm). The target cell samples were pipetted into the disposable counting chambers, which hold precisely 20 µL for image-based cytometric analysis. Four sets of images of brightfield and each fluorescent field (BR, FL1, and FL2) were captured sequentially for each sample and the target cells were identified and analyzed by the software. The Cellometer software scanned each brightfield image and outlined the dark membrane of each cell, in which the fluorescent intensity was measured to generate a fluorescent distribution for the cell population. The results were directly exported to a proprietary file format (.NXDat) for fluorescent analysis with FCS Express™ 4 Flow Cytometry.

Primary immune cell preparation

To isolate B1 B cells, peritoneum washout from 15 to 20 BALB/c or C56BL/6 mice were pooled together. On average, 1×10^6 B220 ^{int} CD5 ^{int} B1 B cells can be sorted from peritoneum washout from 4 to 5 mice. B2 B cells were purified from spleen using MACS beads and column. The isolation and purification procedures were performed according to manufacturer's protocols.

Method validation using LPS stimulation

In order to validate the Cellometer analysis method for detecting cell proliferation, 1–5 µg/mL LPS was used to stimulate proliferation of B1 and B2 B cells [12]. B1 and B2 B cells were isolated from peritoneum and spleen, labeled with carboxy-fluorescein succinimidyl ester (CFSE) and cultured in RPMI 1640 in a standard 96-well microplate in the presence or absence of LPS for 6 days. CFSE staining, which fluorescently labels the free amines of cytoplasmic proteins, does not affect cellular differentiation [13] and is passed on to each daughter generation with attenuating fluorescence [14-16].

Proliferation effect of five TLR agonists on B1 B cells

In order to demonstrate the capability of Cellometer image cytometer, isolated B1 B cells were stimulated with final concentrations of 1 µg/mL of Pam3cys and LPS, 30 µg/mL of PolyIC, 3 µg/mL of CLO97, and 0.5 µg/mL of CpG. A control was established by adding only cell culture medium. The cells were first labeled with CFSE and then cultured in RPMI 1640 with and without the TLR agonists for 7 days.

Cellometer vision cell proliferation kinetic measurement

For method validation, the fluorescence of CFSE-labeled B1 and B2 B cells was measured using Cellometer image cytometer on day 1, 3, 5, and 6. For the proliferation effects from the panel of TLR agonists, the fluorescence of CFSE-stained B1 B cells was measured on day 2, 4, 6, and 7. TLR agonists treated cells and controls were stained with PI on each day, thus the viability of each sample could be monitored during culturing. Two fluorescent optics modules, VB- 535-402 and VB-660-502, were used for CFSE and PI detection, respectively. Fluorescent threshold in the software was set to 0% to measure total fluorescence of each counted cell from the captured images. The fluorescent measurements were exported and analyzed in FCS Express. The results were compared to the flow cytometry data collected on day 7.

Flow cytometry analysis

CFSE fluorescence was acquired using a LSRII flow cytometer on the 6th day of the cell proliferation experiment. A 488 nm laser excitation and a FITC emission filter were used for CFSE fluorescence measurement. Results were analyzed using the FCS Express™ software and compared with Cellometer data.

Results

Method validation.

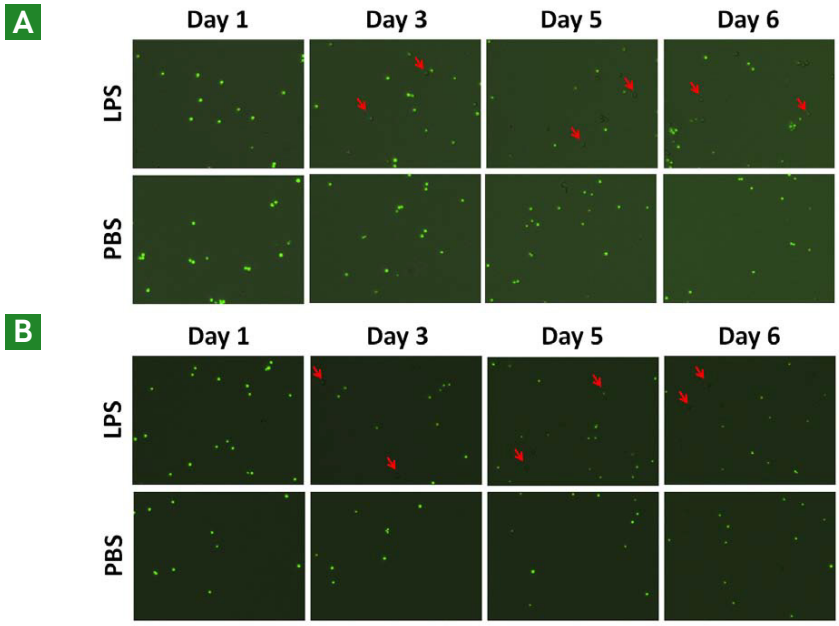


Figure 1: a and b) LPS was used to stimulate proliferation of B1 and B2 B cells. CFSE fluorescent images of LPS-stimulated and PBS-control (A) B1 and (B) B2 B cells on day 1, 3, 5, and 6. Both B1 and B2 B cells showed reduced intensity of green fluorescence (pseudo-colored) as the culturing period increased. In contrast, the PBS control B1 and B2 B cells showed consistent fluorescent signal throughout the culturing period. The red arrows indicate cells with diminished fluorescent signal.

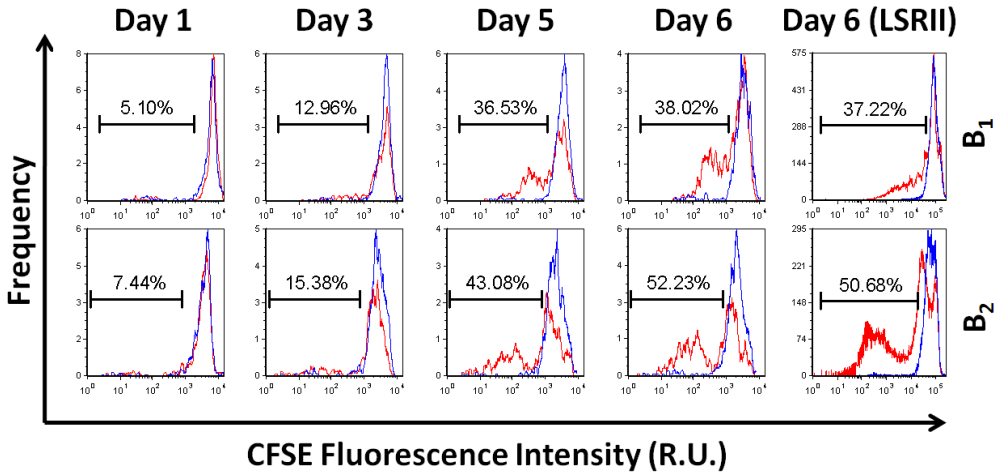


Figure 2: CFSE fluorescence intensity distributions of LPS-stimulated (red) and PBS-control (blue) B1 and B2 B cells on day 1, 3, 5 and 6. The percent divided cells increased from 5.10, 12.96, 36.53, to 38.02% for B1 B cells, and from 7.44, 15.38, 43.08, to 52.23% for B2 B cells. Furthermore, flow cytometry (LSRII) showed comparable results with Cellometer on day 6 for both B1 and B2 B cell proliferation at 37.22 and 50.68%, respectively.

Effect of TLR agonists on B1 B cell proliferation.

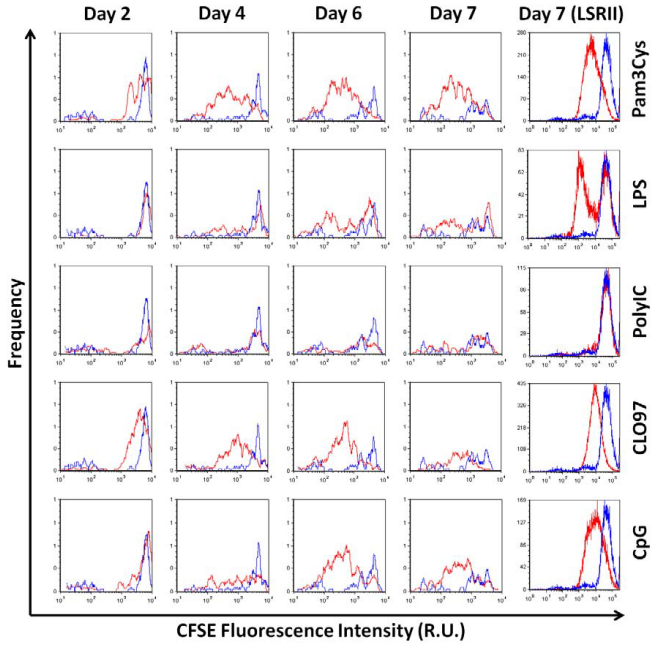


Figure 3: CFSE fluorescent intensity distribution of TLR agonist-stimulated (red) and control (blue) B1 B cells on day 2, 4, 6, and 7. B1 B cells stimulated with Pam3Cys, LPS, CLO97, and CpG showed various degrees of proliferation indicated by the frequency of cells with attenuated fluorescent signal. Only the PolyIC did not induce a clear proliferation of the B1 B cells. LSRII flow cytometer was used to measure the CFSE fluorescence intensity on day 7, which was consistent with the results obtained from Cellometer image cytometer.

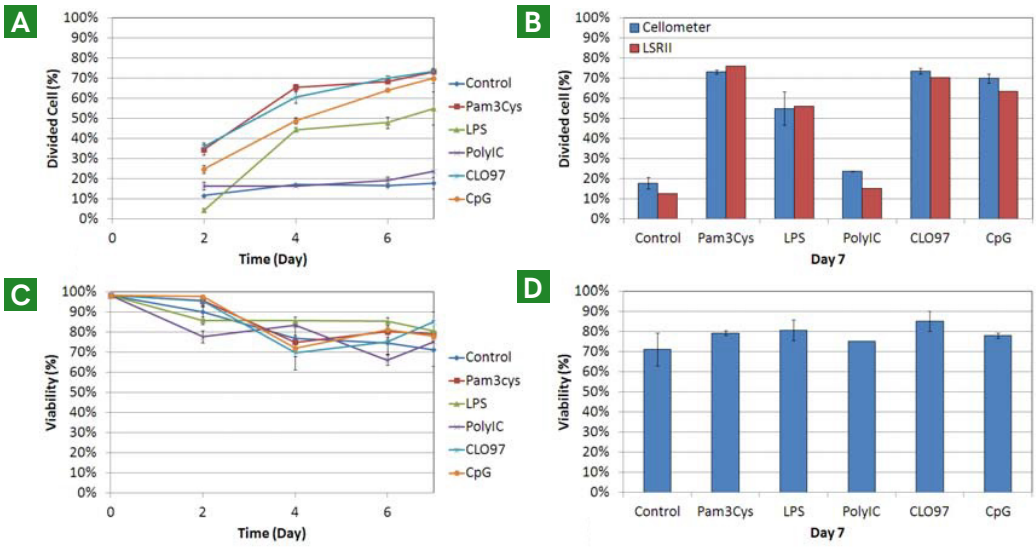


Figure 4: A) Cellometer Vision was able to generate a kinetic plot of proliferation for each TLR agonist. The PolyIC showed similar divided cell percentage as the control, whereas Pam3Cys, LPS, CLO97, and CpG showed a clear increase of divided cells. It is interesting to note that for LPS induction, proliferation of B1 B cells occurred at a later time point than the other TLR agonists. In this case, LPS did not show proliferation until day 4, whereas the Pam3Cys, CLO97, and CpG showed comparable proliferation from day 2.

B) Percentages of divided cells obtained from Cellometer image cytometer were comparable to the LSRII flow cytometer on day 7. The percent divided cells for control, Pam3Cys, LPS, PolyIC, CLO97, and CpG were 12.7, 76.0, 56.2, 15.3, 70.5, and 63.4%, respectively for LSRII, and 17.8, 73.2, 55.0, 23.6, 73.6, and 70.0%, respectively for Cellometer. For each sample, the results were comparable between both systems with a difference of less than 10%.

C, D) Various reductions in viability were measured in each sample through the PI staining method, where the viability of control, Pam3Cys, LPS, PolyIC, CLO97, and CpG were reduced to 71.3, 79.4, 80.8, 75.3, 85.2, and 78.0%, respectively.

Conclusion

- Cellometer image-based cytometry was validated through the detection and analysis of LPS-induced B1 and B2 B cell proliferation with results comparable to those obtained by flow cytometry. The CFSE fluorescent distributions were plotted each day, where attenuated fluorescent signals occurred only after day 3, indicating proliferation of both B1 and B2 B cells.
- Although the number of cells analyzed using Cellometer was approximately 2,000 per sample compared to the 200,000 counts from flow cytometry, the ability to accurately measure cell proliferation was clearly demonstrated. Since flow cytometry requires a large amount of target cell sample, it was difficult to utilize the system to measure proliferation on a day-to-day basis. In contrast, Cellometer required only 20 µL of sample, which can be extracted from the same sample well for each CFSE measurement, thus improving the consistency of kinetic proliferation data generated.
- Multiple samples can be analyzed and compared with similar efficiency using Cellometer. B1 B cells induced with Pam3Cys, LPS, PolyIC, CLO97, and CpG showed various fluorescent attenuation levels for each TLR agonist at different time points. Pam3Cys, CLO97, and CpG-induced proliferation were detected on day 2 of the culturing period, whereas LPS was detected on day 4, which is consistent with previous results.
- In this case, LPS did not show proliferation until day 4, whereas the Pam3Cys, CLO97, and CpG showed comparable proliferation from day 2. These kinetic proliferation results can be used to reduce experimental time and improve assay conditions.
- Because of the low quantity of sorted B1 B cells, most of the isolated cells were reserved for the flow cytometry end-point measurements, which represented approximately 20,000 cells. Therefore, the number of cells available for Cellometer detection was reduced to approximately 500 cells. Even with this low cell count, Cellometer was able to measure proliferation with results comparable to flow cytometry.
- Propidium iodide was concurrently used to measure the viability of each sample. The control and PolyIC showed the lowest viability in comparison to the other TLR agonists that stimulated proliferation. Over the culturing period, the viability of B1 B cells reduced to an average of ~77 ± 8%, where the CLO97-induced sample showed the highest viability on day 7, indicating that CLO97 may have allowed the B1 B cells to remain viable for a longer period of time compared to the other TLR agonists.
- We have developed a novel kinetic proliferation analysis method using Cellometer image-based cytometry. The Cellometer system has demonstrated improvements over conventional flow cytometry methods including low sample volume requirement, the ability to generate brightfield and fluorescent images, the use of LEDs that eliminates the need for precise optical alignment, and the lack of fluid handling that eliminates the issue of clogging.
- The cell proliferation analysis method described here would require only 20 µL/sample (at least 500 cells), thus much fewer mice would need to be sacrificed and more experimental conditions could be tested. Further improvement on the system can be focused on increasing the imaging area for higher cell counts to analyze other rare cell types like regulatory T cells, which can further advance the capability of the system.

References

1. Assy, N. and G.Y. Minuk, *Liver regeneration: methods for monitoring and their applications*. Journal of Hepatology, 1997. 26: p. 945-952.
2. Colozza, M., et al., *Proliferative markers as prognostic and predictive tools in early breast cancer: where are we now?* Annals of Oncology, 2005. 16: p. 1723-1739.
3. Chan, L.L., et al., *A rapid detection method for apoptosis and necrosis measurement using the Cellometer imaging cytometry.* Apoptosis : an international journal on programmed cell death, 2011. 16(12): p. 1295-303.

4. Chan, L.L., et al., *Direct concentration and viability measurement of yeast in corn mash using a novel imaging cytometry method.* J Ind Microbiol Biotechnol, 2011. 38(8): p. 1109-15.

5. Chan, L.L., et al., *Cellometer vision as an alternative to flow cytometry for cell cycle analysis, mitochondrial potential, and immunophenotyping.* Cytometry. Part A : the journal of the International Society for Analytical Cytology, 2011. 79(7): p. 507-17.

6. Robey, R.W., et al., *Rapid detection of ABC transporter interaction: potential utility in pharmacology.* J Pharmacol Toxicol Methods, 2011. 63(3): p. 217-22.

7. Hayashi, E.A., S. Akira, and A. Nobrega, *Role of TLR in B Cell Development: Signaling through TLR4 Promotes B Cell Maturation and Is Inhibited by TLR2.* Journal of Immunology, 2005. 174: p. 6639-6647.

8. Lathia, J.D., et al., *Toll-Like Receptor 3 Is a Negative Regulator of Embryonic Neural Progenitor Cell Proliferation.* Journal of Neuroscience, 2008. 28(51): p. 13978-13984.

9. Oberg, H.-H., et al., *Regulation of T cell activation by TLR ligands.* European Journal of Cell Biology, 2011. 90: p. 582-592.

10. Peng, S.L., *Signaling in B cells via Toll-like receptors.* Current Opinion in Immunology, 2005. 17: p. 230-236.

11. Uccellini, M.B., et al., *Autoreactive B Cells Discriminate CpG-Rich and CpG-Poor DNA and This Response Is Modulated by IFN-α.* Journal of Immunology, 2008. 181: p. 5875-5884.

12. Ding, H., et al., *Blockade of B Cell-Activating Factor Suppresses Lupus-like Syndrome in Autoimmune BXSB Mice.* Journal of Cellular and Molecular Medicine, 2009. 14(6B): p. 1717-1725.

13. Hasbold, J., et al., *Quantitative analysis of lymphocyte differentiation and proliferation in vitro using carboxyfluorescein diacetate succinimidyl ester.* Immunology and Cell Biology, 1999. 77: p. 516-522.

14. Lyons, A.B., *Divided we stand: Tracking cell proliferation with carboxyfluorescein diacetate succinimidyl ester.* Immunology and Cell Biology, 1999. 77: p. 509-515.

15. Lyons, A.B., *Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution.* Journal of Immunological Methods, 2000. 243: p. 147-154.

16. Lyons, A.B. and C.R. Parish, *Determination of lymphocyte division by flow cytometry.* Journal of Immunological Methods, 1994. 171: p. 131-137.

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